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Characterization of C3 receptors on cultured rat glomerular endothelial cells

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Characterization of C3 receptors on cultured rat glomerular endothelial cells. In this study we characterized C3 receptors on cultured rat glomerular endothelial cells (GEnC), using immunochemical and molecular techniques. GEnC membrane proteins were immunoprecipitated with a polyclonal antibody directed towards mouse complement receptor 2 (CR2). This anti-MCR2 immunoprecipitated GEnC proteins of 120 and 150 kDa. By immunohistochemistry, anti-MCR2 stained GEnC in rat glomeruli *in vivo*. Given the presence of CR2-like proteins on GEnC, subsequent studies were done to determine whether GEnC had C3-binding proteins. GEnC proteins of 80, 200, and 300 kDa specifically bound to columns of rat C3d-Sepharose and C3b-Sepharose, illustrating that these proteins were binding to the C3d portion of C3. The 80, 200, and 300 kDa C3d-binding proteins were distinct from the 120 and 150 kDa anti-MCR2 reactive proteins, as shown by immunoabsorption studies. Next, a specific cDNA probe for rat CR2 was generated by RT-PCR. Oligonucleotides were chosen from highly conserved regions in mouse and human CR2 spanning 224 bases, with the rationale that these would also be conserved in the rat. A 224 bp PCR product was generated from both rat GEnC and rat kidney cDNA, illustrating the presence of CR2 mRNA in these tissues. By Northern analysis, the CR2 PCR product hybridized to mRNA of 2 and 5 kb from GEnC. The 5 kb transcript was also identified in rat kidney mRNA. Therefore, proteins immunologically related to mouse CR2 are present in GEnC *in vitro* and *in vivo*. C3d-binding proteins of 80, 200, and 300 kDa are also present on rat GEnC, yet these appear to be immunologically distinct from the proteins identified by anti-MCR2. Whether the GEnC CR2 mRNA transcripts of 2 and 5 kb are translated into the 80 and 200 kDa C3d-binding proteins or the 120 and 150 kDa mouse CR2-like proteins remains to be defined.

In humans, complement receptor 1 (CR1) and CR2 are members of the regulators of complement activation gene cluster, and have primary specificity for C3b and C3d, respectively [1, 2]. Proteins in this family share a common 60 amino acid short consensus repeat (SCR), containing a framework of four cysteine residues, along with other conserved amino acids [2, 3]. This common ancestral precursor appears to have been duplicated many times, and is present in other complement proteins (C1r, C1s, C2, and factor B) as well as other non-complement proteins (such as, interleukin-2 receptor, factor XIII subunit) [2]. Of the nonhuman species, complement receptors in the mouse have been the most completely elucidated. Mouse CR1 and CR2 are encoded by alternatively spliced *Cr2* gene transcripts [4–7]. Consequently, both proteins share 15 SCRs, and both are recognized by monoclonal and polyclonal anti-CR2 antibodies [8, 9].

Human CR1 has a fairly wide distribution and is present on B- and T-lymphocytes, monocytes, macrophages, neutrophils, erythrocytes, and glomerular epithelial cells. In contrast, human CR2 has a more restricted distribution, being located on B-lymphocytes, a subset of T-lymphocytes, epithelial cells, and follicular dendritic cells [10–12]. Similar to human CR2, both mouse CR1 and CR2 have a limited distribution, and are expressed solely on B-lymphocytes and follicular dendritic cells [13]. In neither species have CR1 or CR2 been identified on endothelial cells.

In the mouse and rat, proteins that bind C3b are present on unstimulated neutrophils and platelets, yet they are not CR1 or CR2 [8, 14]. In recent studies, we have identified two C3b-binding proteins of 80 and 200 kDa on rat platelets and neutrophils, named C3bR-80 and C3bR-200 [14]. These were not immunoprecipitated by antibodies to human or mouse CR1/CR2. Therefore, these proteins most likely serve as the rat immune adherence receptors. From the available information, C3bR-80 and C3bR-200 appear to be distinct from CR1 and CR2. Still, since the cDNA of these proteins remain uncloned, this remains speculative.

In the course of our studies on complement regulators and receptors in rat glomerular cells [15, 16], we noted that polyclonal anti-mouse CR2 antibodies (anti-MCR2) reacted with cultured rat glomerular endothelial cells (GEnC). We were therefore interested in further characterizing C3d receptors on GEnC, using immunochemical and molecular techniques.

Methods

Antibodies

An *Escherichia coli* trpE-mouse CR2 fusion protein, containing SCRs 1-10, was used to make rabbit anti-MCR2 antibodies as described previously [7]. Anti-MCR2 IgG was isolated by protein G-Sepharose affinity chromatography (Pharmacia, Piscataway, NJ, USA). Anti-human C3d was obtained from Dako (Carpinteria, CA, USA), and cross-reacts with rat C3d [17].

Affinity columns

Rat C3 was isolated as described previously [18]. Briefly, rat C3 was purified by a 5 to 11% polyethylene glycol precipitation

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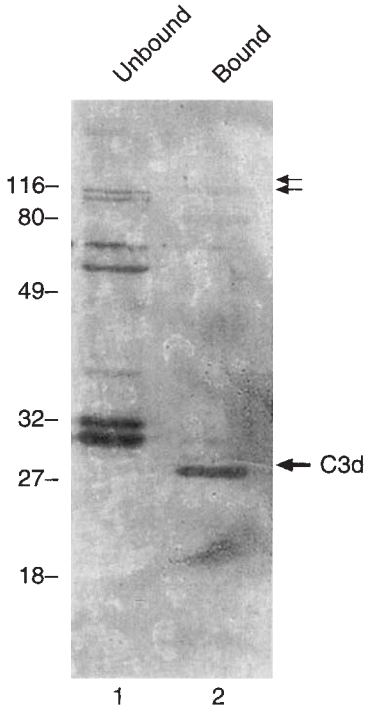

Fig. 1. *Western blot analysis of C3d-Sepharose with anti-C3d.* C3 fragments remaining unbound (lane 1) and bound (lane 2) to C3d-Sepharose were analyzed by reducing SDS-PAGE followed by immunoblotting with anti-human C3d.

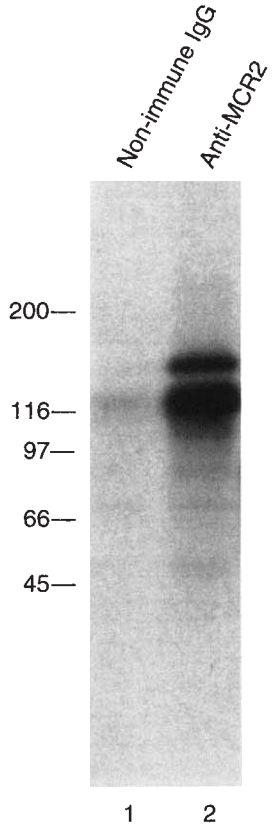

Fig. 2. *Immunoprecipitation of GEnC membrane proteins with anti-MCR2.* Surface radiolabeled GEnC were immunoprecipitated with anti-MCR2 (lane 2) or nonimmune IgG (lane 1), separated by reducing SDS-PAGE, and subjected to autoradiography.

followed by Mono Q anion exchange chromatography (Pharmacia). The resulting preparation was pure by SDS-PAGE. C3d was generated by trypsinization (10% wt/wt, 3 hr, 37°C) and bound via its free sulfhydryl group to thiopropyl-Sepharose (Pharmacia). The supernatant that was not bound to thiopropyl-Sepharose was collected. From an aliquot of C3d-Sepharose, bound C3d was released from the beads by incubation in 1 mM 2-mercaptoethanol. In this C3d-Sepharose column, the C3 α chain was present only as the 30 kDa C3d fragment as shown by Western blotting [18] with anti-C3d (Fig. 1, Lane 2, large arrow). Not bound to Sepharose were C3c fragments (Lane 1), as well as small amounts of intact α and α' chains (small arrows). Rat C3b-Sepharose was prepared as described previously [18].

As a control column, 42 mg BSA (Sigma Chemical Co., St. Louis, MO, USA) was bound to 1.5 gm CNBr-Sepharose (Pharmacia) according to the manufacturer's instructions.

GEnC culture

Rat GEnC were cultured as described elsewhere [19]. Cells were grown on culture dishes coated with fibronectin (Collaborative Research, Bedford, MA, USA) in RPMI-1640 medium (Life Technologies, Gaithersburg, MD, USA) with 10% fetal bovine serum and 10% Nu-Serum (Collaborative Research). GEnC were characterized by their morphology, the ability to take up di-acetyl LDL, and the possession of angiotensin converting enzyme activity [19]. Cells were used between passages 16 and 20.

Immunohistochemistry

Normal rat kidneys were snap frozen in isopentane on dry ice. Five micrometer sections were cut and fixed in 4% paraformaldehyde. Endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol and sections were blocked in 10% normal goat serum. Tissue was then sequentially incubated in anti-MCR2 IgG or normal rabbit IgG as a control (0.1 mg/ml), biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA), ABC development reagent (Vector), and diaminobenzidine. Sections were then counterstained with alcian blue and methyl green, and dehydrated in graded ethanols.

Immunoprecipitations

GEnC in culture dishes were surface radiolabeled with ^{125}I using lactoperoxidase and H_2O_2 as previously described [16]. Following labeling, cells were incubated in phosphate buffered saline (PBS) containing 1 mM EDTA for 20 minutes at room temperature with periodic agitation, and cells not detached after this time were removed from the substratum with a rubber policeman. Cells were washed with PBS, and then solubilized for 15 minutes on ice in 1 ml PBS, pH 7.2, containing 10 mM EDTA, 10 mM iodoacetamide (Sigma), 5 mM diisopropylfluorophosphate (Sigma), 1% NP-40. Nuclei and cytoplasmic debris were pelleted at 14,000 × g. Unincorporated $Na^{125}I$ was removed by Sephadex G-25 chromatography (Pharmacia). By this technique, 4 to 6 ×

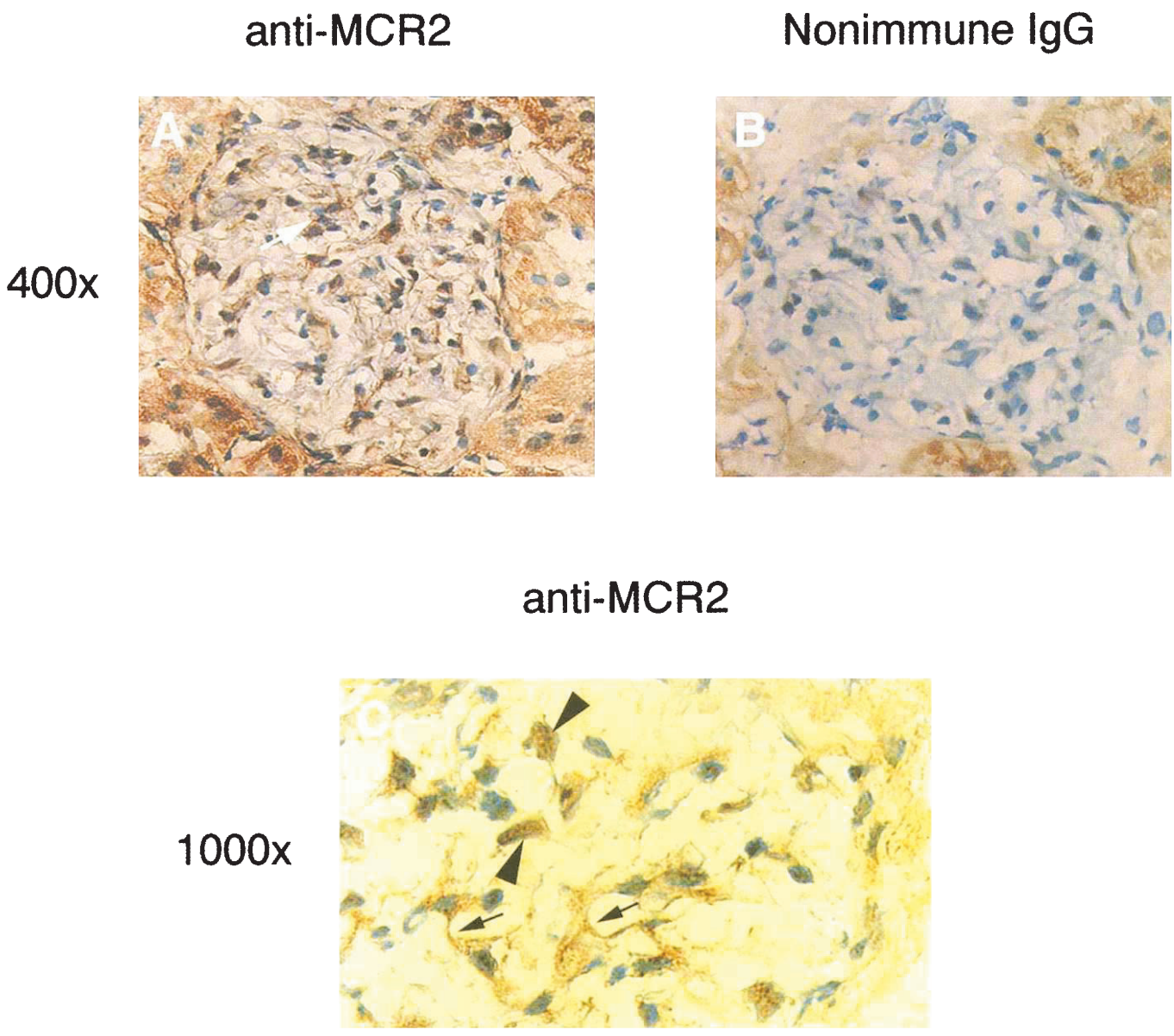

Fig. 3. *Immunoperoxidase staining for CR2 in normal rat kidney.* Sections were incubated with anti-MCR2 IgG (**A**, ×400; **C**, ×1000) or with nonimmune IgG (**B**, ×400). Publication of this figure in color was made possible by a grant from Hoechst Marion Roussel, Kansas City, MO, USA.

10^6 trichloroacetic acid precipitable cpm were obtained per culture dish.

After preclearing of antigens with normal rabbit serum and protein G-Sepharose, antigens were immunoprecipitated with 200 μg rabbit anti-MCR2 IgG or 200 μg nonimmune rabbit IgG as a control, followed by addition of 50 μl protein G-Sepharose (50% vol/vol). After thorough washing with 0.6 M NaCl, 0.05 M Tris-HCl, pH 7.5, 0.2% NP-40, immunoprecipitated proteins were subjected to SDS-PAGE and autoradiography.

Affinity chromatography

For C3d affinity chromatography, solubilized ^{125}I-labeled GEnC membrane proteins were diluted with H_2O and PBS to final NaCl and NP-40 concentrations of 0.05 M and 0.1%, respectively. Low ionic strength is necessary because of the low affinity interactions between complement receptors and C3 fragments [18, 20]. These preparations were applied to BSA-Sepharose and C3d-Sepharose columns in series. The BSA-Sepharose column was used to exclude nonspecifically bound proteins from the C3d-Sepharose column. Once samples had passed through the BSA-Sepharose column, it was disconnected. Columns were washed with ≈ 50 column volumes of 0.05 M NaCl, 0.01 M Na phosphate, pH 7.2, 0.1% NP-40. Specifically bound material was eluted with 0.5 M NaCl, 0.02 M Na phosphate, pH 7.2, 0.1% NP-40, and analyzed by SDS-PAGE and autoradiography. C3b affinity chromatography was performed similarly.

In a group of experiments designed to evaluate whether proteins were binding specifically to C3d-Sepharose, ^{125}I-labeled

GEnC membrane proteins were diluted to final NaCl and NP-40 concentrations of 0.05 M and 0.1%, respectively. The sample was divided in half and applied to identically sized columns of BSA-Sepharose or C3d-Sepharose. The columns were washed and specifically bound proteins eluted as before. Equivalent volumes of eluted material were compared by SDS-PAGE and autoradiography.

To immunoabsorb GEnC proteins reactive with anti-MCR2 prior to C3d affinity chromatography, ^{125}I-labeled membrane proteins were first incubated with 1.0 mg anti-MCR2 or normal rabbit IgG as a control, and then incubated with 0.7 ml protein G-Sepharose (50% vol/vol). The supernatant was then diluted to 0.05 M NaCl and 0.1% NP-40, and subjected to C3d affinity chromatography as described above. Proteins immunoprecipitated by anti-MCR2 or nonimmune IgG contained in the protein G pellets were processed as detailed in the previous section.

Erythrocyte rosetting assay

Sheep erythrocytes (E) bearing C3b (EC3b) were prepared as described previously [21]. By this technique, C3 was treated with trypsin to generate C3b, which also leads to the production of smaller amounts of C3bi [18]. The protocol described by Kasinath et al to evaluate complement receptors in glomerular epithelial cells was followed [22]. Briefly, confluent GEnC were washed three times with Hank's balanced salt solution (HBSS), and then incubated with EC3b. As a control, E not bearing C3b were used. E or EC3b were added at 1×10^7/ml in HBSS and incubated with GEnC for 60 minutes at 37°C with continuous shaking. Cells were washed three times with HBSS, and the numbers of E or EC3b per high powered field (×200) were counted under an inverted phase contrast microscope. Data were compared by unpaired *t*-testing.

Reverse transcription (RT) and the polymerase chain reaction (PCR)

Total RNA from GEnC and normal rat kidney were isolated by a single-step guanidinium isothiocyanate-phenol-chloroform extraction (Tri-Reagent; MRC, Inc., Cincinnati, OH, USA). Poly(A^+) RNA was purified by affinity chromatography on oligo-dT cellulose columns (MRC). Mouse spleen poly(A^+) RNA was obtained from Clontech (Palo Alto, CA, USA). Genomic DNA from rat and mouse liver were isolated by treatment of tissues with SDS and proteinase K, followed by phenol/chloroform/isoamyl alcohol extractions [23].

Highly conserved areas in the nucleotides that encode the eleventh and twelfth SCRs of both human and murine CR2 (spanning 224 bases) were used to design oligonucleotide primers for PCR. The sequences of the 5′ sense primer was (5′-TGTCAAAATGCTGAGAATGGGAC-3′), and that of the 3′ antisense primer was (5′-CTCCAAGAGCCATGACCTTTAGAA-3′), both of which correspond to the mouse sequence. cDNA was produced from 1 μg poly (A^+) mRNA by RT using random hexamers (SuperScript Preamplification System; GIBCO BRL). Subsequent PCR was performed in tubes containing RT-generated cDNA or genomic DNA, 50 mM KCl, 20 mM Tris-HCl, 2.5 mM $MgCl_2$, 100 μg/ml BSA, 100 μM of each deoxynucleotide triphosphate, 0.1 μM of each primer, and 2.5 U *Taq* polymerase. Thirty cycles of a one minute denaturation at 94°C, one minute annealing at 60°C, and one minute extension at 72°C were performed. The products thus formed were electrophoresed through a 1.5% agarose gel and stained with ethidium bromide. The specific rat CR2 PCR product (**Results**) was purified by preparative agarose gel electrophoresis and extracted from the gel with a commercially obtained kit (MERMAID; Bio 101, La Jolla, CA, USA).

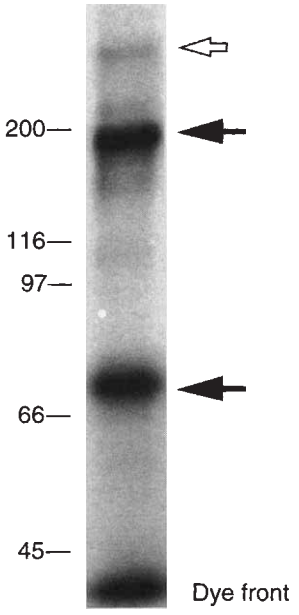

Fig. 4. *C3d affinity chromatography of GEnC membrane proteins.* Surface radiolabeled GEnC were subjected to C3d affinity chromatography, followed by reducing SDS-PAGE and autoradiography.

Northern analyses

One microgram of poly(A^+) RNA from cultured GEnC or normal rat kidney was electrophoresed through a denaturing agarose gel. RNA was transferred by capillary action to a nylon membrane and cross-linked to the membrane by ultraviolet irradiation. The specific CR2 cDNA probe and a β-actin cDNA [24] were random primer labeled with [α-^{32}P]dCTP. Hybridization and washing were performed under high stringency as described previously [24].

Results

Reactivity of anti-MCR2 with GEnC in vitro and in vivo

While studying complement receptors in cultured rat glomerular cells, we included anti-MCR2 in our immunoprecipitation analyses. Surprisingly, anti-MCR2 specifically immunoprecipitated GEnC proteins of 120 and 150 kDa as determined by reducing SDS-PAGE (Fig. 2, lane 2). Under non-reducing conditions, these two migrated slightly faster, at calculated molecular weights of 115 and 145 kDa, respectively (not shown). The 150 kDa protein is consistent with the sizes of human and mouse CR2 [5, 7].

By immunohistochemistry, anti-MCR2 clearly reacted with intrinsic glomerular cells (Fig. 3). The cytoplasm of GEnC appeared to be stained (Fig. 3C, arrows). Furthermore, in some

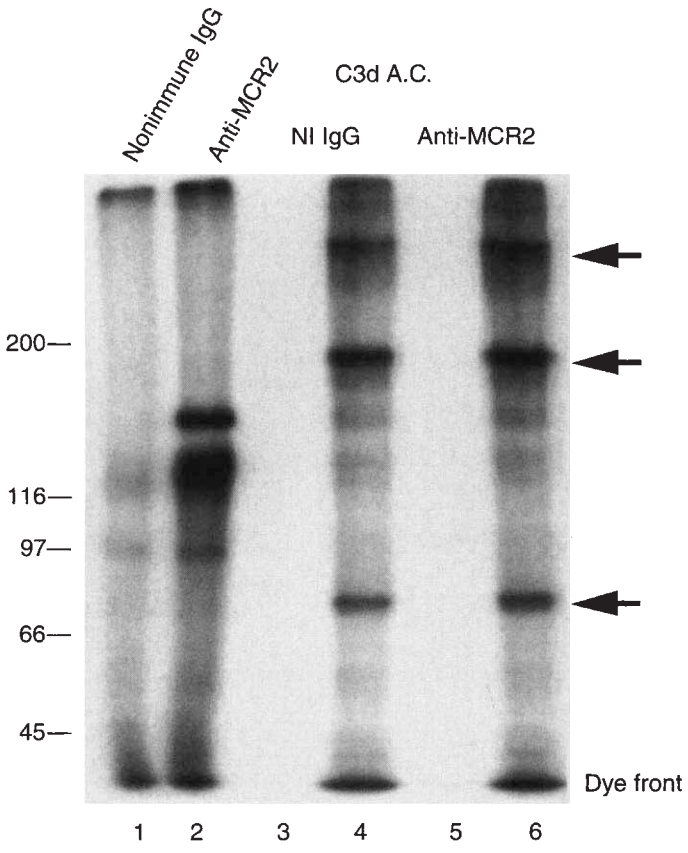

Fig. 5. *Immunoabsorption of GEnC membrane proteins prior to C3d affinity chromatography.* Surface radiolabeled GEnC were immunoabsorbed with nonimmune IgG (lane 4) or anti-MCR2 IgG (lane 6) prior to C3d affinity chromatography. Proteins immunoprecipitated in this experiment by nonimmune IgG (lane 1) and anti-MCR2 (lane 2) were electrophoresed on the same reducing SDS-polyacrylamide gel.

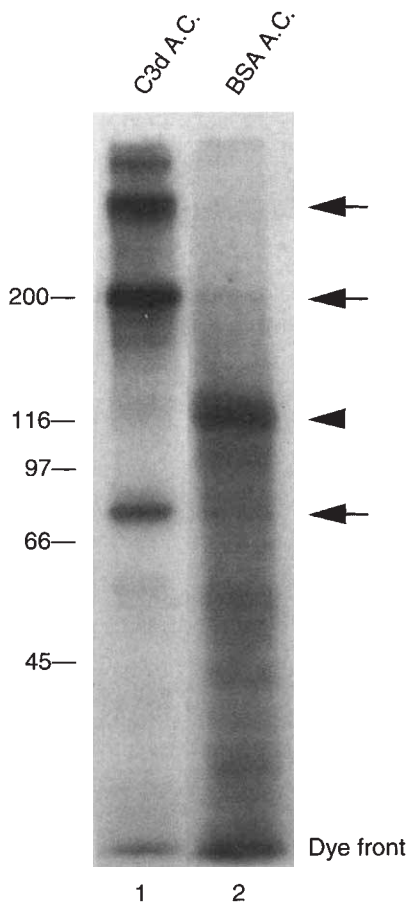

Fig. 6. *Comparison of GEnC membrane proteins bound to C3d-Sepharose with those bound to BSA-Sepharose.* Surface radiolabeled GEnC membrane proteins were subjected to affinity chromatography on C3d-Sepharose (lane 1) or BSA-Sepharose (lane 2). Proteins eluted with high salt buffer from the columns were electrophoresed on a reducing SDS-polyacrylamide gel followed by autoradiography. Both lanes were run on the same gel, but have been spliced for this analysis.

cells, staining over nuclei was evident (Fig. 3C, arrowheads). There was also focal mesangial cell staining apparent (Fig. 3A, white arrows). These data show that GEnC *in vitro* and *in vivo* contain proteins reactive with anti-MCR2.

C3d affinity chromatography

Given the presence of CR2-like proteins on GEnC, we were interested to determine whether these proteins bound C3d. By C3d affinity chromatography, proteins of 80 and 200 kDa were recovered from GEnC (as shown by reducing SDS-PAGE, Fig. 4, arrows). These findings were reproducible in four separate experiments. In addition, a C3d-binding protein with an approximate molecular weight of 300 kDa was identified that had variable intensity in different experiments (Fig. 4, open arrow). These three C3d-binding proteins bound equivalently to C3b-Sepharose (not shown), illustrating that these proteins were binding to the C3d portion of C3b.

We performed further studies to characterize these C3d-binding proteins more completely. Although their molecular weights suggested they were distinct from the 120 and 150 kDa proteins immunoprecipitated by anti-MCR2, we conducted experiments to confirm that these two groups of proteins were unrelated. Radiolabeled GEnC proteins were first immunoabsorbed with anti-MCR2 or normal rabbit IgG prior to C3d affinity chromatography. As shown in Figure 5, proteins of 80, 200, and 300 kDa were recovered by C3d affinity chromatography in GEnC proteins first absorbed with nonimmune rabbit IgG (lane 4, arrows), comparable to our previous results. Preabsorption with anti-MCR2 did not affect the subsequent recovery of proteins from the C3d-Sepharose column (lane 6). For purposes of comparison, proteins immunoprecipitated by nonimmune IgG and anti-MCR2 in these experiments are shown in lanes 1 and 2, respectively. In addition, the fractions collected from the C3d-Sepharose columns following washing but immediately before elution are shown in lanes 3 (nonimmune IgG preabsorption) and 5 (anti-MCR2 IgG preabsorption). These confirm that the identified C3d-binding proteins were specifically eluted from the column with high salt buffer. Therefore, the 80, 200, and 300 kDa C3d-binding proteins of GEnC are immunologically distinct from those proteins identified by anti-MCR2.

When the C3d-binding proteins were subjected to SDS-PAGE under non-reducing conditions, they migrated at calculated molecular weights of 65, 150, and ≈ 250 kDa (not shown). These data

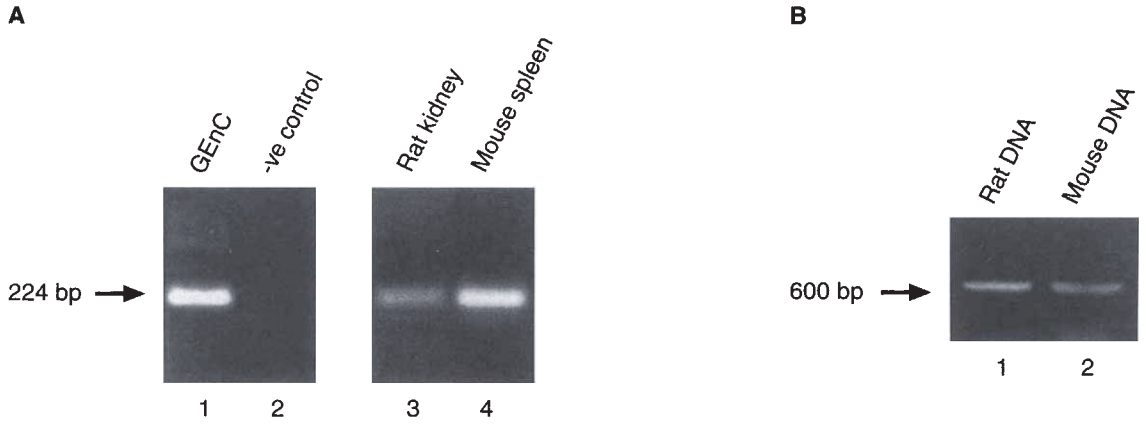

Fig. 7. *RT-PCR analysis using CR2 oligonucleotide primers.* **A.** Poly(A^+) RNA from GEnC (lane 1), rat kidney (lane 3), or mouse spleen (lane 4) were subjected to RT-PCR using oligonucleotide primers chosen from conserved regions in human and mouse CR2 separated by 224 bases. In lane 2 is the negative control that lacked RNA, but was otherwise treated identically. **B.** PCR was also performed on rat (lane 1) and mouse (lane 2) genomic DNA using these CR2 primers.

are consistent with the presence of intrachain disulfide bonds, characteristic of the SCR-containing complement receptors [1]. Thus, while these proteins are not identified by anti-MCR2, their capacity to bind C3d and their decreased apparent molecular weight under non-reducing conditions is consistent with SCR-containing proteins.

We needed to exclude that nonspecific binding to C3d-Sepharose could account for our results. Therefore, the following studies compared proteins that bound specifically to C3d-Sepharose with those that bound to BSA-Sepharose, a technique used by others to validate specificity of binding to C3 columns [25–28]. Eluted from the BSA-Sepharose column were a number of faintly visible protein bands from GEnC, along with a prominent 120 kDa protein (Fig. 6, lane 2, arrowhead), the identity of which is unknown. In contrast, the 80, 200, and 300 kDa proteins were strongly present by autoradiography after elution from the C3d-Sepharose column (lane 1, arrows). In the experiments shown in Figures 5 and 6, we observed a large molecular weight protein by C3d affinity chromatography, which conceivably represents aggregates of the C3 binding proteins. Therefore, the three C3d-binding proteins of 80, 200, and 300 kDa do not appear to bind Sepharose in a nonspecific fashion.

To establish the significance of GEnC C3 binding proteins, studies were performed to evaluate whether these cells were capable of binding C3b on sheep E. In these studies, GEnC bound 314.1 ± 34.1 EC3b versus 58.7 ± 7.3 E per high-powered field (mean ± SEM, $N = 9$, $P < 0.001$). Therefore, the identified C3 receptors of GEnC bind particles bearing C3 fragments, such as may occur in immune complexes.

RT-PCR and Northern analysis

Oligonucleotide primers for PCR were chosen from two highly conserved regions in human and mouse CR2 that were separated by 224 bases in both species with the rationale that these would also be conserved in the rat. As shown by the arrow in Figure 7A, a 224 bp PCR product was generated from rat GEnC cDNA (lane 1) and normal rat kidney cDNA (lane 3) using these primers. These were identical in size to that generated in parallel from mouse spleen cDNA (lane 4). PCR was also performed on rat and mouse genomic DNA. By this approach, a 600 bp PCR product was generated from mouse DNA (Fig. 7B, lane 2), consistent with the known presence of an intron between SCRs 11 and 12 [7]. The same sized product was also generated from rat genomic DNA (lane 1). Thus, the rat and mouse appear to have comparable mRNA and genomic DNA for CR2 at SCRs 11 and 12.

The 224 bp rat GEC PCR product was purified by preparative agarose gel electrophoresis, ^{32}P labeled, and then used in Northern analyses. As shown in Figure 8, this CR2 probe hybridized to 2 and 5 kb mRNA bands from cultured GEnC (arrows, lane 1). The 5 kb mRNA band was also present in RNA from rat kidney. Therefore, cultured rat GEnC contain CR2 mRNA. CR2 mRNA is also present *in vivo* in normal kidney.

Discussion

Here we show the presence of five apparently novel proteins on cultured GEnC. Two proteins are reactive with antibodies raised to recombinant mouse CR2. At least one of these proteins is present *in vivo* as shown by our immunohistochemical data. The other three proteins are C3d-binding proteins that are immunochemically distinct from the proteins identified by anti-MCR2. We also show that GEnC have mRNA with 224 nucleotides that have similarity to that of mouse CR2. These 224 bases appear to be contained in mRNA transcripts of 2 and 5 kb, as shown by our Northern analysis data. The 5 kb CR2 mRNA is also present *in vivo*.

The identity of the 120 and 150 kDa proteins reactive with anti-MCR2 is confusing. We have shown a nearly identical immunoprecipitation profile from rat neutrophils using anti-MCR2, while rat erythrocytes do not have either protein [14]. These neutrophil proteins bound C3b-Sepharose to a minor extent [14]. This may reflect that the column contained C3bi, as an inevitable contaminant of rat C3b preparation [18], to which CR2 can bind [29, 30]. In ongoing studies, we have also shown that mouse platelets contain large quantities of 125 and 150 kDa proteins identified by anti-MCR2. These proteins bound to mouse C3d-Sepharose [31]. This background information supports that

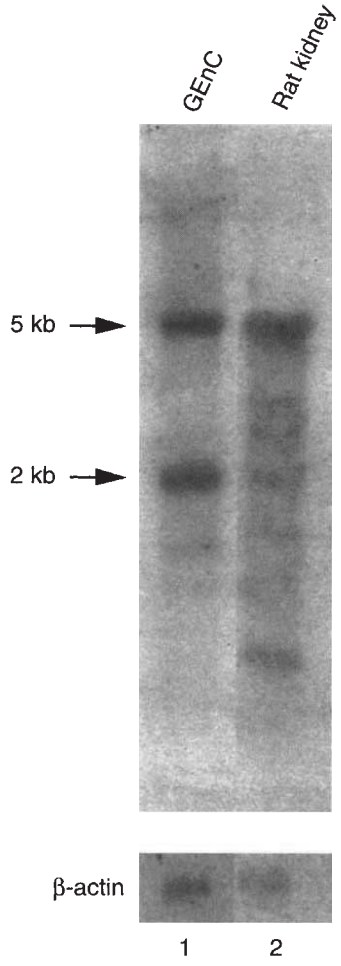

Fig. 8. *Northern analysis of GEnC and rat kidney RNA using the rat CR2 RT-PCR product.* Following autoradiography, the membrane was stripped and reprobed with a β-actin cDNA. Both lanes were run on the same gel, but have been spliced for this analysis.

the proteins identified by anti-MCR2 are relevant to the complement system. Based upon our data in other cells, the interaction of these proteins with C3 fragments may be of such a low affinity that we were unable to document C3d binding in these GEnC proteins.

The presence of 80 and 200 kDa C3d binding proteins in GEnC is reproducible. The finding of a 300 kDa C3d binding protein is also consistent, although its intensity varies. Our observations cannot be attributable to nonspecific binding to Sepharose affinity columns under low ionic strength conditions (0.05 M NaCl), as shown by our experiments using BSA-Sepharose. We also conclusively show that these proteins are unrelated to those identified by anti-MCR2. Similar to the findings with anti-MCR2, a nearly identical profile was seen in rat neutrophils using C3b affinity chromatography, even down to the observation that the 300 kDa product was variably present in different neutrophil isolations [14]. Because we used a single line of GEnC, we can't attribute this variation to genotypic differences, and may simply reflect minor experimental variations from day-to-day. It is possible that the 80 and 200 kDa C3d- and C3b-binding GEnC proteins we have identified here are the same as the neutrophil proteins we have termed C3bR-80 and C3bR-200, respectively [14]. In those studies, we did not determine if the C3b-binding proteins also bound C3d.

It is worth noting that human and mouse CR3 and CR4, which are members of the integrin superfamily, are C3 binding proteins [29]. While the molecular weights of 80 and 200 kDa could be consistent with the β and α chains, respectively, of either CR3 or CR4, these proteins are not rat CR3 or CR4 for the following reasons. First, the C3d column did not contain any C3bi, to which CR3 and CR4 specifically bind. Second, we performed C3d affinity chromatography in the presence of EDTA, which eliminates the divalent cation-dependent binding of CR3 and CR4 to C3bi [29]. The fact that the GEnC proteins bound to C3d as well as to C3b indicates that at least one binding site for these proteins resides in the C3d portion of C3, as with human and mouse CR1 and CR2 [30, 32–34]. The C3 binding and apparent presence of intrachain disulfide bonds are consistent with these C3d-binding proteins containing the basic SCR repeating structure common to mouse and human C3 binding proteins. Proof for this will require cloning the cDNA for these proteins.

To identify mRNA for rat CR2, we chose a highly conserved area in human and mouse CR2 to design primers for RT-PCR. This was a successful approach, and illustrated that rat GEnC contain mRNA with similarities to those encoding human and mouse CR2. That this is relevant *in vivo* comes from the finding that an identically sized RT-PCR product was generated from rat kidney. By Northern blotting we confirmed the presence of CR2 mRNA in cultured GEnC and in the kidney *in vivo*. It is worth noting that the CR2 PCR product was generated from SCRs 11–12 of CR2, while the anti-MCR2 antibody used here is reactive towards SCRs 1-10, so a direct link between the immunoprecipitation and Northern analysis data cannot be made. It does seem likely that the 2 and 5 kb CR2 mRNA transcripts are translated into anti-MCR2 reactive proteins, but this also requires proof.

Until now, the only C3-binding cell type in the glomerulus was believed to be the visceral glomerular epithelial cell. Observations made twenty years ago placed C3 receptors on this cell in humans [35], which later was identified as CR1 [36]. Kasinath et al identified C3b and C3d receptors on cultured rat glomerular epithelial cells [22], and in later studies, we identified a 200 kDa C3b-binding protein on these cells [18]. Cultured GEnC can now be added to the list as a glomerular cell type that has C3 receptors.

Largely through the work of Sacks and Daha, and their colleagues, it is becoming increasingly apparent that intrinsic glomerular cells are capable of synthesizing complement components [37–42]. Although this has the potential to be injurious to cells under certain conditions, it seems likely that complement production by glomerular cells serves a useful purpose. Given that the glomerulus is constantly exposed to immune complexes, production of complement by intrinsic cells can facilitate dissolution of immune complexes [43]. To this end, cultured GEnC also produce C3 (S. Adler, S.H. Sacks, unpublished observations). Once C3b is deposited on immune complexes, either from fluid phase or locally produced C3, and further cleaved to C3bi and C3d by circulating factor I, a mechanism should exist for subsequent metabolism of these complexes. The presence of C3 receptors on GEnC provides one such mechanism for the removal of potentially inflammatory immune complexes.

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References

1. Hourcade D, Holers VM, Atkinson JP: The regulators of complement activation (RCA) gene cluster. *Adv Immunol* 45:381–416, 1989
2. Campbell RD, Law SKA, Reid KBM, Sim RB: Structure, organization, and regulation of the complement genes. *Ann Rev Immunol* 6:161–195, 1988
3. Klickstein LB, Wong WW, Smith JA, Weis JH, Wilson JG, Fearon DT: Human C3b/C4b receptor (CR1): Demonstration of long homologous repating domains that are composed of the short consensus repeats characteristic of C3/C4 binding proteins. *J Exp Med* 165:1095–1112, 1987
4. Kurtz CB, Paul MS, Aegerter M, Weis JJ, Weis JH: Murine complement receptor gene family. II. Identification and characterization of the murine homologue (Cr2) to human CR2 and its molecular linkage to Crry. *J Immunol* 143:2058–2067, 1989
5. Fingeroth JD, Benedict MA, Levy DN, Strominger JL: Identification of murine complement receptor type 2. *Proc Natl Acad Sci USA* 86:242–246, 1989
6. Kurtz CB, O'Toole E, Christensen SM, Weis JH: The murine complement receptor gene family. IV. Alternative splicing of Cr2 gene transcripts predicts two distinct gene products that share homologous domains with both human CR2 and CR1. *J Immunol* 144:3581–3591, 1990
7. Molina H, Kinoshita T, Inoue K, Carel JC, Holers VM: A molecular and immunochemical characterization of mouse CR2. Evidence for a single gene model of mouse complement receptors 1 and 2. *J Immunol* 145:2974–2983, 1990
8. Kinoshita T, Takeda J, Hong K, Kozono H, Sakai H, Inoue K: Monoclonal antibodies to mouse complement receptor type 1 (CR1). Their use in a distribution study showing that mouse erythrocytes and platelets are CR1-negative. *J Immunol* 140:3066–3072, 1988
9. Molina H, Wong W, Kinoshita T, Brenner C, Foley S, Holers VM: Distinct receptor and regulatory properties of recombinant mouse complement receptor 1 (CR1) and Crry, the two genetic homologues of human CR1. *J Exp Med* 175:121–129, 1992
10. Holers VM, Kinoshita T, Molina H: The evolution of mouse and human complement C3-binding proteins: Divergence of form but conservation of function. *Immunol Today* 13:231–236, 1992
11. Dovezenski N, Billetta R, Gigli I: Expression and localization of proteins of the complement system in human skin. *J Clin Invest* 90:2000–2012, 1992
12. Birkenbach M, Tong X, Bradbury LE, Tedder TF, Kieff E: Characterization of an Epstein-Barr virus receptor on human epithelial cells. *J Exp Med* 176:1405–1414, 1992
13. Kinoshita T, Fujita T, Tsunoda R: Expression of complement receptors CR1 and CR2 on murine follicular dendritic cells and B lymphocytes, in *Dendritic Cells in Lymphoid Tissues*, edited by Imai Y, Tew JG, Hoefsmit ECM, Amsterdam, Basel, Elsevier Science Publishers B.V., 1991, p 271
14. Quigg RJ, Holers VM: Characterization of rat complement receptors and regulatory proteins. CR2 and Crry are conserved, and the C3b receptor of neutrophils and platelets is distinct from CR1. *J Immunol* 155:1481–1488, 1995
15. Quigg RJ, Holers VM, Morgan BP, Sneed AE: Crry and CD59 regulate complement in rat glomerular epithelial cells and are inhibited by the nephritogenic antibody of passive Heymann nephritis. *J Immunol* 154:3437–3443, 1995
16. Quigg RJ, Morgan BP, Holers VM, Adler S, Sneed AE, Lo CF: Complement regulation in the rat glomerulus: Crry and CD59 regulate complement in glomerular mesangial and endothelial cells. *Kidney Int* 48:412–421, 1995
17. Schulze M, Pruchno CJ, Burns M, Baker PJ, Johnson RJ, Couser WG: Glomerular C3c localization indicates ongoing immune deposit formation and complement activation in experimental glomerulonephritis. *Am J Pathol* 142:179–187, 1993
18. Quigg RJ, Galishoff ML, Sneed AE, Kim D: Isolation and characterization of complement receptor type 1 (CR1) from rat glomerular epithelial cells. *Kidney Int* 43:730–736, 1993
19. Adler S, Eng B: Integrin receptors and function on cultured glomerular endothelial cells. *Kidney Int* 44:278–284, 1993
20. Kinoshita T, Lavoie S, Nussenzweig V: Regulatory proteins for the activated third and fourth components of complement (C3b and C4b) in mice. II. Identification and properties of complement receptor type 1 (CR1). *J Immunol* 134:2564–2570, 1985
21. Quigg RJ: Inhibition of the alternative pathway of complement by glomerular chondroitin sulphate proteoglycan. *Immunology* 76:373–377, 1992
22. Kasinath BS, Maaba MR, Schwartz MM, Lewis EJ: Demonstration and characterization of C3 receptors on rat glomerular epithelial cells. *Kidney Int* 30:852–861, 1986
23. Strauss WM: Preparation of genomic DNA from mammalian tissues, in *Current Protocols in Molecular Biology* (Unit 2.2), edited by Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, New York, John Wiley & Sons, Inc., 1996
24. Quigg RJ, Sneed AE: Molecular characterization of rat glomerular epithelial cell complement receptors. *J Am Soc Nephrol* 4:1900–1908, 1994
25. Dykman TR, Cole JL, Iida K, Atkinson JP: Polymorphism of human erythrocyte C3b/C4b receptor. *Proc Natl Acad Sci USA* 80: 1698–1702, 1983
26. Cole JL, Housely GA Jr, Dykman TR, MacDermott RP, Atkinson JP: Identification of an additional class of C3-binding membrane proteins of human peripheral blood leukocytes and cell lines. *Proc Natl Acad Sci USA* 82:859–863, 1985
27. Manthei U, Nickells MW, Barnes SH, Ballard LL, Cui WY, Atkinson JP: Identification of a C3b/iC3 binding protein of rabbit platelets and leukocytes. A CR1-like candidate for the immune adherence receptor. *J Immunol* 140:1228–1235, 1988
28. Birmingham DJ, Cosio FG: Characterization of the baboon erythrocyte C3b-binding protein. *J Immunol* 142:3140–3144, 1989
29. Ross GD: Complement receptors, in *Encyclopedia of Immunology*, edited by Roitt IM, Delves PJ, London, Academic Press, 1992, p 388
30. Pramoonjago P, Takeda J, Kim YU, Inoue K, Kinoshita T: Ligand specificities of mouse complement receptors types 1 (CR1) and 2 (CR2) purified from spleen cells. *Int Immunol* 5:337–343, 1993
31. Quigg RJ, Holers VM: Characterization of complement receptors on mouse neutrophils and platelets. (abstract) *Mol Immunol* 33(Suppl 1):64, 1996
32. Molina H, Kinoshita T, Brenner Webster C, Holers VM: Analysis of C3b/C3d binding sites and factor I cofactor regions within mouse complement receptors 1 and 2. *J Immunol* 153:789–795, 1994
33. Fishelson Z: Complement C3: A molecular mosaic of binding sites. *Mol Immunol* 28:545–552, 1991
34. Koistinen V, Wessberg S, Leikola J: Common binding region of complement factors B, H and CR1 on C3b revealed by monoclonal anti-C3d. *Complement Inflamm* 6:270–280, 1989
35. Shin ML, Gelfand MC, Nagle RB, Carlo JR, Green I, Frank MM: Localization of receptors for activated complement on visceral epithelial cells of the human renal glomerulus. *J Immunol* 118:869–873, 1977
36. Kazatchkine MD, Fearon DT, Appay MD, Mandet C, Bariety J: Immunohistochemical study of the human glomerular C3b receptor in normal kidney and in seventy-five cases of renal diseases. *J Clin Invest* 69:900–912, 1982
37. Sacks SH, Zhou W, Pani A, Campbell RD, Martin J: Complement C3 gene expression and regulation in human glomerular epithelial cells. *Immunology* 79:348–354, 1993

38. ZHOU W, CAMPBELL RD, MARTIN J, SACKS SH: Interferon-gamma regulation of C4 gene expression in cultured human glomerular epithelial cells. *Eur J Immunol* 23:2477–2481, 1993
39. SACKS S, ZHOU W, CAMPBELL RD, MARTIN J: C3 and C4 gene expression and interferon-gamma-mediated regulation in human glomerular mesangial cells. *Clin Exp Immunol* 93:411–417, 1993
40. SACKS SH, ZHOU W, ANDREWS PA, HARTLEY B: Endogenous complement C3 synthesis in immune complex nephritis. *Lancet* 342:1273–1274, 1993
41. TIMMERMAN JJ, VERWEIJ CL, VAN GIJLSWIJK-JANSSEN DJ, VAN DER WOUDE FJ, VAN ES LA, DAHA MR: Cytokine-regulated production of the major histocompatibility complex class-III-encoded complement proteins factor B and C4 by human glomerular mesangial cells. *Hum Immunol* 43:19–28, 1995
42. VAN DEN DOBBELSTEEN ME, VERHASSELT V, KAASHOEK JG, TIMMERMAN JJ, SCHROEIJERS WE, VERWEIJ CL, VAN DER WOUDE FJ, VAN ES LA, DAHA MR: Regulation of C3 and factor H synthesis of human glomerular mesangial cells by IL-1 and interferon-gamma. *Clin Exp Immunol* 95:173–180, 1994
43. SCHIFFERLI JA, NG YC, PETERS DK: The role of complement and its receptor in the elimination of immune complexes. *N Engl J Med* 315:488–495, 1986